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A new subtraction technique for molecular cloning of rare antiviral antibody specificities from phage display libraries

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SUMMARY

The preparation of random combinatorial libraries exposed on the surface of phage provides a route for the selection of diverse high affinity human monoclonal antibodies. However, in particular settings, the isolation of genes coding for a rare antibody can be elusive because some epitopes are predominant and because, in the case of impure antigens, the protein or any compound of interest can be present in relatively minimal amount. In this paper, we describe the successful utilization of a new strategy of "pre-adsorption" panning that allowed us to clone a rare human monoclonal antibody fragment and to access a different antibody repertoire. The procedure is easy, fast, inexpensive, can be used together with other panning techniques and can be particularly useful in cloning antibodies against rare or unknown determinants.

Key-words: Combinatorial library, Phage display; Human monoclonal antibodies.

INTRODUCTION

The preparation of random combinatorial libraries exposed on the surface of phage provides a route for the selection of diverse high affinity human monoclonal antibodies (HuMAbs). The application of this technique made possible the production of an extremely large range of HuMAbs against a plethora of viral antigens (Burton, D.R. *et al.*, 1991; Zebedee, S.L. *et al.*, 1992; Williamson, R.A. *et al.*, 1993; Burioni, R. *et al.*, 1994; Plaisant, P. *et al.*, 1997). Sometimes, the isolation of a given Fab antibody fragment can be particularly difficult because some epitopes are predominant and because, in the case of impure antigens, the protein or any compound of interest can be present in minor quantity. Many techniques have been developed for overcoming this problem, like epitope masking (Ditzel *et al.*, 1995) or sandwich-capture (Sanna *et al.*, 1995) procedures. However, these approaches require the availability of a mAb against the antigen or epitope of interest. In this paper, we describe the successful utilization of a new procedure of preadsorption panning that allowed us to clone a rare human monoclonal antibody fragment specific for herpes simplex virus type 1 (HSV-1). The procedure is easy, fast, inexpensive and can be used together with the previously mentioned panning techniques.

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MATERIALS AND METHODS

The preparation of human antibody Fab libraries displayed on the surface of M13 phage was carried out as described (Burton *et al.*, 1991; Williamson *et al.*, 1993). Construction of this library has been described (Cattani *et al.*, 1995). The 50-year-old subject had a clinical history of labial herpetic infection and never had signs or symptoms of genital infection. The library, evaluated as described (Burton *et al.*, 1991; Williamson *et al.*, 1993) resulted in approx. 4×10^6 members. The library was stored as phage suspension at −70°C until use.

Antigen binding phages were selected against ELISA wells coated with lysates of VERO cells infected with HSV-1 (Behring) by a panning procedure already described (Burton *et al.*, 1991).

In the case of subtraction panning, the experiment was carried out identically, except that 200 µl of phage suspension were overlaid on ten HSV-2 antigen-coated ELISA wells and incubated for 2 h at 37°C before being exposed to the HSV-1 antigen-coated wells. After this step, 140 µl of the recovered phage were subjected to immunoaffinity enrichment against HSV-1 antigens as described above. Also in this case, the number of phage eluted during the procedure was monitored as described.

Soluble Fab preparations and ELISA tests were carried as already described (Williamson *et al.*, 1993). Optical densities at 450 nm (OD_{450}) were read after 30 min of incubation at room temperature in the dark. Each Fab was also tested against uninfected cell lysates. This value was held as blank. All Fabs were tested at least in duplicate. Clones were held as positive when the OD_{450} value was more than 0.8.

Indirect immunofluorescence was carried out as described (Cattani *et al.*, 1995). Slides were observed and independently assessed by two observers. Fluorescence was scored in an arbitrary scale ranging from 0 to ++++. Discordances between the two observations are reported in brackets. All samples were run at least in duplicate. HSV-1- and HSV-2-infected cells were also checked for infection using commercially available mouse mAb (Kallergen) following manufacturer's directions. All the recombinant Fabs were also tested against uninfected Vero cells under the same conditions.

Nucleic acid sequencing was performed with a 373A automated DNA sequencer (Applied Biosystems) using a Taq fluorescent dideoxynucleotide terminator cycle sequencing kit (Applied Biosystems). Primers used for the elucidation of heavy chain sequence have been previously described (Burioni *et al.*, 1994).

RESULTS

The phage display Fab library obtained from an immune donor repertoire was challenged against the HSV-1 and HSV-2 antigens as described. After five rounds of panning, selected phages were converted to a soluble Fab expressing phagemid system. *E. coli* XL-1 blue strain transformed with Fab expression vector was used as a source for the Fab molecules for further characterization.

Indirect immunofluorescence analysis performed with the Fabs on different strains of HSV-1 and HSV-2 showed that positive Fabs derived from the "non-preadsorbed panning" experiment were 18 out of 20 clones. All positive clones were able to strongly recognize both strains of HSV. Clones were also tested in ELISA against the antigens used for selection, showing positivity with a large range of O.D. values. All the clones reacted similarly with HSV-1 and HSV-2 antigens (table I). The sequence analysis of the heavy chain of this set of Fabs (table Ia) showed the prevalence of one particular DNA sequence among the positive clones. IIF patterns are consistent with data obtained by sequence analysis (data not shown). Minimal sequence differences between some clones (e.g. 4 and 5) can be due to reverse transcription or *Taq* polymerase errors.

Characterization of the set of 20 Fabs obtained from the "preadsorbed panning" experiment showed a very different pattern (table Ib); 9 of the 20 clones obtained were positive when analysed in IFA against HSV-1- and HSV-2-infected cells. In this case, however, 8 clones did not react when tested against the HSV-2 strain, while strongly recognizing cells infected with several strains of HSV-1. The antibody preparation was extensively tested against several clinical isolates of HSV-1 and HSV-2, yielding reproducible results. The same antibody turned out to be very effective for the laboratory diagnosis of the infection of a cell monolayer by HSV-1 (R. Burioni *et al.*, submitted). While all the clones reacting with both viruses were also positive in ELISA assay, some clones reacting only with HSV-1 gave ELISA results that were indeterminate with both antigens (table II). It is worth noting that even after preadsorption, there were some Fabs that reacted with HSV-2, and one of the clones present in the non-preadsorbed set was present, even if less represented, also in the "preadsorbed" group of Fabs. Sequence analysis showed that all the clones specifically recognizing HSV-1 shared the same heavy chain sequence. Seven of the clones that recognized both HSVs appeared to have the same sequence of the majority of the positive Fabs of the previous set. Two other clones reacting with both viruses appeared to have a completely different sequence that was not found among the clones of the first set.

DISCUSSION

The utilization of combinatorial libraries displayed on the surface of filamentous phage gives the researcher a very handy tool for selection. However,

Table I. Reactivity and sequences of anti-HSV Fabs obtained from the immunoaffinity selection (panning) without preadsorption.

Fab n.	ELISA HSV-1	ELISA HSV-2	IFA HSV-1	IFA HSV-2	Heavy chain CDR3 aa sequence
1	+	+	+++	+++	QRTHSRVTYFAASEFDP
2	+	+	+++(+)	+++	QRTHSRVTYFAASEFDP
3	–	–	–	–	GTLAYYYTWTY
4	+	+	+++	+++(+)	QRTHSRVGYFAASEFDP
5	+	+	++	+++	QRTHSRVTYFAASEFDP
6	–	–	–	–	DKYDFDY
7	+	+	+++	+++	QRTHSRVTYFAASEFDP
8	+	+	++++	+++	QRTHSRVTYFAASEFDP
9	+	+	+++	+++	QRTHSRVTYFAASEFDP
10	+	+	++(+)	+++	QRTHSRVTYFAASEFDP
11	+	+	++	++	QRTHSRVTYFAASEFDP
12	+	+	++	+++	QRTHSRVTYFAASEFDP
13	+	+	+(+)	+++	QRTHSRVTYFAASEFDP
14	+	+	++	++	QRTHSRVTYFAASEFDP
15	+	+	+++	+++	QRTHSRVTYFAASEFDP
16	+	+	+++	++++	QRTHSRVTYFAASEFDP
17	+	+	++	+++	QRTHSRVTYFAASEFDP
18	+	+	+++	++	QRTHSRVTYFAASEFDP
19	+	+	++	+++	QRTHSRVTYFAASEFDP
20	+	+	++++	+++(+)	QRTHSRVTYFAASEFDP

Table II. Reactivity and sequences of anti-HSV Fabs obtained from the immunoaffinity selection (panning) with preadsorption.

Fab n.	ELISA HSV-1	ELISA HSV-2	IFA HSV-1	IFA HSV-2	Heavy chain CDR3 aa sequence
1	+	+	++++	+++	QRTHSRVTYFAASEFDP
2	+	+	+++	–	ARYSYGLFDS
3	±	±	++(+)	–	ARYSYGLFDS
4	±	±	+++(+)	–	ARYSYGLFDS
5	+	+	++	++	QRTHSRVTYFAASEFDP
6	–	–	–	–	DANCSYYFDY
7	±	±	++	–	ARYSYGLFDS
8	+	+	++	+	QRTHSRVTYFAASEFDP
9	±	±	+++	–	ARYSYGLFDS
10	–	–	–	–	DRTVFGSLDY
11	+	+	++(+)	+(+)	QRTHSRVTYFAASEFDP
12	–	–	–	–	DKHSLRRFGFWNGLHHSGFGHYHMDV
13	+	+	++++	++++	QRTHSRVTYFAASEFDP
14	±	+	++	–	ARYSYGLFDS
15	±	+	+	+	SKMSYLALDY
16	±	+	+	+	SKMSYLALDY
17	+	+	++	++	QRTHSRVTYFAASEFDP
18	±	±	+++	–	ARYSYGLFDS
19	+	+	++	++	QRTHSRVTYFAASEFDP
20	±	+	+++	–	ARYSYGLFDS

in some circumstances the isolation of Fabs recognizing non-immunodominant epitopes by panning against a mixture of antigens can remain elusive, and in our experience many attempts toward cloning HSV-1-specific human recombinant Fabs did not yield satisfactory results, despite a vigorous screening effort. The procedure described in this work, with a very simple additional step, increased the efficiency of cloning of these rare specificities, offering an additional contribution to the full exploitation of the combinatorial library-phage display technique.

Even if this technique appears to be very useful in practical approaches, one caveat the researcher must keep in mind is that, as shown clearly by our results, the subtraction of unwanted clones is only partial, and should be considered only as a "negative enrichment" rather than a complete subtraction. In spite of this limitation, we think that this new strategy can give a considerable contribution to the comparative studying of the humoral immune response and to the isolation of antibodies directed against rare or non-immunodominant specificities.

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